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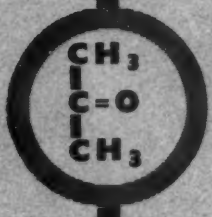

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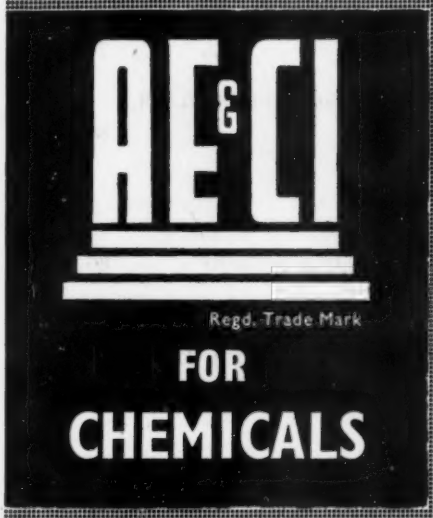

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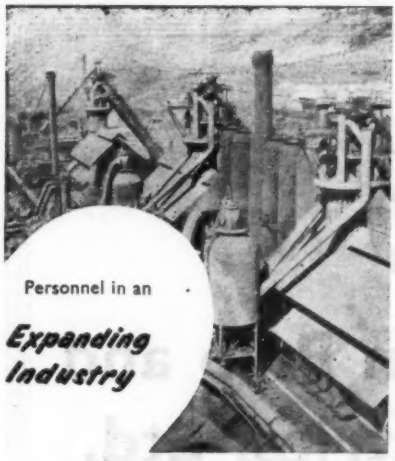

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A NEW MANOMETRIC APPARATUS AND ITS USE IN SEWAGE FERMENTATION STUDIES

by

A. W. M. COAKER and K. A. MURRAY

OPSOMMING

'n Nuwe manometriese apparaat is ontwikkel wat groot genoeg is vir toetsoplossings om met standaard hoeveelhede van rioolwaters geinokuleer te word. Die bestek en sensitiwiteit is groter as dié van standaard manometriese apparate. Oplossings van ureum, stysel, glukose, gelatien, eiwit, natriumstearaat en suspensies van stysel en cholesterol is met monsters van rioolwaters geinokuleer en die suurstofverbruik bepaal.

SUMMARY

A new manometric apparatus large enough for test solutions to be seeded with inoculants of sewage liquors and with a range and sensitivity greater than those of standard manometric apparatus, is described. Solutions of urea, starch, dextrose, gelatin, egg albumen, sodium stearate and suspensions of starch and cholesterol are inoculated with samples of sewage liquors and the resulting oxygen uptake studied.

To investigate respiration in solutions of pure compounds seeded with bacteria, a sensitive apparatus which can follow oxygen uptake over a considerable range is desirable. Adeney[1] with his "long aeration" apparatus achieved the required range by entrapping some air in his flasks when the supply of dissolved oxygen was inadequate. Gotaas[2] used a large number of different B.O.D. dilutions, but this had the disadvantage of introducing the irrelevant factor of different dilutions, the precise effect of which on the rates of oxygen uptake determined was either unknown or an undesirable complication. Caldwell and Langelier[3] fitted 125 ml. reaction flasks to Warburg manometers and by varying their sample volumes between 10 and 75 ml. were able to determine manometrically the B.O.D.'s of various liquors and ranges from 0-500 p.p.m. to 0-35 p.p.m. with manometer sensitivities varying between 2·0 p.p.m. of B.O.D. per mm. scale division, the sensitivity being inverse to the range. This point was further discussed by Ludwig *et al.*[4] who stated that for most B.O.D. testing a 50-100 ml. flask was most practical.

The standard Barcroft manometer has two severe limitations, *viz.* flasks much larger than 40 ml. cannot be used and the length of the scale is fixed and may be too short for some purposes. The former factor was found to be a serious drawback in work on respiration in solutions of known compounds, involving the use of very small inoculants of sewage liquor (*ca.* 1 drop) or else aliquots of highly diluted sewage liquors. The reproducibility attained was poor and seemingly due to variations from inoculant to inoculant. Consequently, a much larger manometer and scale was constructed to ensure sensitivity over a wide range so that sufficiently large amounts of inoculant could be used.

EXPERIMENTAL

The apparatus. The apparatus, based on the principle of the Barcroft respirometer, is shown in fig. 1. A volume of about 600 ml. of test liquor was chosen. The three-necked flasks (1 litre) were maintained at 37° in a water thermostat. The manometer, made of 0·5 mm. capillary tubing, had a fluid reservoir similar to the standard arrangement for Warburg manometers, fitted at the bottom and was charged with coloured liquid paraffin. The "zero" level of the paraffin could be controlled by a screw clip. Narrow bore rubber pressure tubing, coated with several applications of rubber cement, was used for connections.

Absorption of carbon dioxide was accomplished by rolls of filter paper impregnated with 20 *per cent.* potassium hydroxide (1·5 ml.) in absorption tubes supported as shown in fig. 1 by glass tubes. Holes were made in the latter tubes for the introduction of different atmospheres.

The apparatus was checked regularly for leaks by placing distilled water in both flasks, sucking the paraffin up in the right hand manometer link, closing the taps and running the apparatus overnight. Movement of the manometer fluid of ss than 2 mm. was taken as evidence that there were no leaks.

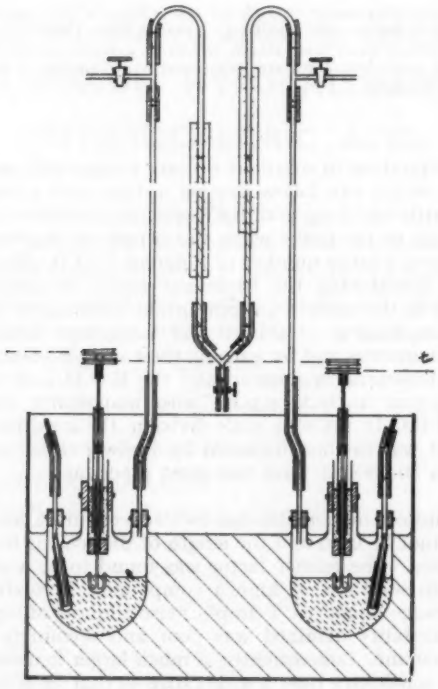

FIG. 1

Calibration. The Munzer and Neumann method was used. Volumes of air were withdrawn from the right hand flask into a burette and the manometric differences read after half-an-hour. The results of 5 determinations of the manometer constant gave a coefficient of variability of 0·9 *per cent.* Using approximate volumes and the Dixon formula with altitude correction, the calculated value for Ko_2 was 1·4 *per cent.* lower. The mean value of Ko_2 obtained by the Munzer and Neumann determination was 31·8 μl./mm.

Constants previously determined for Barcroft manometers of standard pattern were of the order of 2·6 to 3·2 μl./mm. Thus for standard manometers with flask volumes between 30 and 40 ml., the constants were of the order of 3 μl./mm. for a 5 ml. volume of test liquor, whereas for the new apparatus the constant was of the order of 30 μl./mm. for a 600 ml. volume of the test liquor. Thus it was possible with the new apparatus to use 100 times the volume of test liquor with only a tenfold decrease in sensitivity. Since it was possible to measure manometer differences up to 70 cm. on the new apparatus, for a given sensitivity the range of the new apparatus was about 3·5 times that of the Barcroft manometer.

Measurement of oxygen uptake. Steaming out the flasks followed by flushing with sterile tap water ensured sufficient sterility to prevent oxygen absorption from sterile solutions of the substances tested if rigid sterile precautions were observed in introducing the solutions into the flasks.

Six hundred ml. of the test solution or suspension were used in each case and the volume of inoculant so chosen, in most cases, that it alone produced relatively small manometric effects. The solutions were so made up that the uninoculated solutions produced either small or no manometric effects within the periods of test. When the solutions were made up with distilled water the oxidative bacteria of the inoculants were relatively inactive; tap water was therefore used.

Fermentation of the following substances was investigated: urea, starch, dextrose, gelatin, egg albumen, sodium stearate and cholesterol. These represented substances and types of compound likely to be found in sewage which was obtained from the Cydna Sewage Works, Johannesburg.

Urea. Concentrations of urea in tap water and distilled water ranging from 0·1 to 1·0 *per cent.* were inoculated with 5 to 10 ml. of tank effluent, primary and secondary effluent. The curves (manometric differences *vs.* time) obtained indicated that fast nitrification or nitrosification did not take place under the conditions investigated. For concentrations of urea above 1 *per cent.* fermentation proceeded slowly, but at lower concentrations there was an activating effect. Using concentrations of urea between 0·01 and 0·25 *per cent.* and 10 ml. tank effluent the lag or induction period was about 4 hours. With inoculants of primary and secondary effluents the fermentation proceeded at a slow but fairly steady rate.

Starch. 1 *per cent.* Starch solution inoculated with tank effluent gave rise to a fairly rapid oxygen uptake after a lag period of 8 to 12 hours. Bacteria capable of metabolizing starch are thus present in settled sewage. With primary effluent, after lags of 3 to 6 hours, the oxygen uptake proceeded slightly more slowly than with tank effluent. Curves obtained on inoculation of starch solutions with laboratory sand-filtered secondary effluent indicated a lag of at least 14 hours, whereafter the reaction differed little from that for primary effluent inoculation.

Dextrose. With dextrose concentrations of 0·5 to 1·0 *per cent.* and 1 ml. tank effluent lags of about 16 hours were noted, followed by almost straight line curves of comparatively small slope, showing that the oxidation of dextrose was slower than that of starch. 1 *per cent.* Dextrose solutions inoculated with 10 ml. primary effluent showed a lag of about 12 hours followed by almost straight line graphs with nearly twice the slope obtained with tank effluent inoculant. The base curve for the primary effluent alone, however, showed a lag of only 2 hours. The dextrose must therefore have inhibited the autoxidation of the primary effluent.

It appeared that organisms capable of oxidizing dextrose were present in secondary effluent (sand filtered in the laboratory), but that there was a considerable lag period followed by slow reactions of almost constant rate, this rate being markedly increased by increasing the dextrose concentration.

Gelatin. With 1 ml. of tank effluent in a 0·5 *per cent.* gelatin solution prepared with sterile tap water, the solution having at no stage been heated above 40°, an uptake of oxygen started immediately and proceeded at a fairly constant rate for about 15 hours. Thereafter, the rate of oxygen uptake increased sharply.. With 10 ml. of primary effluent and laboratory sand-filtered secondary effluent almost identical results were obtained.

Egg albumen. It appeared that there were micro-organisms capable of oxidizing egg albumen present in all three of the sewage liquors, those in the laboratory sand-filtered effluent being less active than those in either tank or primary effluent.

Sodium stearate. 0·1 and 0·01 *per cent.* Sodium stearate solutions with 1 ml. of tank effluent gave small manometric differences over 90 hours. These curves were slightly above the base curve for the inoculant alone in sterilized tap water and showed a remarkable correspondence of slight crests and valleys. The peculiar form of these curves was accurately reproduced in further experiments. The time between successive valleys was about 24 hours and the experiments were started at approximately the same time of day, the valleys corresponding to periods of daylight and the crests to darkness. Threads of greenish biological material appeared to be formed during the runs. In view of the correlation between darkness or daylight and rise or fall in the curves, it is possible that photosynthesis or light sterilization influenced gas evolutions.

0·01 *per cent.* Sodium stearate and 10 ml. of primary effluent gave a steadily increasing manometer difference, suggesting that micro-organisms capable of oxidizing sodium stearate were present in primary effluent. Sand-filtered secondary effluent in sterilized tap water gave a curve similar to that obtained with tank effluent.

Cholesterol. Aerobic sewage bacteria did not readily oxidize cholesterol suspended in tap water.

Department of Chemistry and Chemical Engineering,
University of the Witwatersrand,
Johannesburg.

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A MANOMETRIC METHOD FOR DISTINGUISHING BETWEEN DILUTED SEWAGE LIQUORS AND NITRIFIED EFFLUENTS

by

A. W. M. COAKER and K. A. MURRAY

OPSOMMING

'n Metode word beskryf om tussen verdunde rioolwaters en genitrifiseerde afloopwaters te onderskei deur die suurstof verbruik te bepaal van monsters waartoe McConkeysop en nitrietoplossing gevoeg is. Wanneer McConkeysop met natrium touroglikocholaat vervang word is die sensitiviteit van die toets tot verdunnings van 1 in 80 verbeter.

SUMMARY

A method for distinguishing between diluted sewage liquors and nitrified effluents involving the oxygen uptake of samples to which McConkey Broth and nitrite solution are added is described. When McConkey Broth is replaced by sodium tauroglycocholate the sensitivity of the test is increased to dilution of 1 in 80.

The aim of this investigation was to evolve an accurate test for detecting sewage pollution in a sewage works effluent discharged into a stream, either by examination of the effluent itself or of the water downstream. It is not possible to detect by one of the ordinary methods of sanitary analysis relatively small amounts of untreated sewage in nitrified effluents or to distinguish between diluted samples of untreated and treated (nitrified) sewage. The available tests[1,2] include "oxygen absorbed" from acid potassium permanganate, "oxygen consumed" by the dichromate reflux method, B.O.D., "relative stability", free and saline ammonia, albuminoid nitrogen, total nitrogen, organic carbon, bacterial count and B. coli enumeration.

The "oxygen absorbed" or "oxygen consumed" test affords merely a rough index of oxidizable carbonaceous matters present in water and is subject to many limitations such as interference by nitrite and hydrogen sulphide. These two tests do not differentiate between stable and unstable organic matter. The B.O.D. test is based on dissolved oxygen uptake in 5 days at a standard temperature (commonly measured by the Winkler method[3]), but is not capable of distinguishing between much diluted sewage and nitrified effluent. The "relative stability" test involves the use of the redox indicator methylene blue which is reduced to the colourless leuco base by the hydrogen sulphide which accompanies the establishment of putrescible conditions. Thus, provided the total available oxygen (dissolved oxygen, nitrite and nitrate) is sufficient to prevent putrescibility the presence of untreated sewage is not revealed. With the remaining tests, results for diluted sewage samples might readily be equated to nitrified effluents in many instances, thereby affording no distinguishing characteristic other than relative concentration of organic matter and bacteria.

There is much evidence for the presence of enzymes in sewage and that enzymic activity plays an important part in purification processes, e.g. sludge digestion,

biological oxidation by filter beds and the activated sludge process. Since change in enzymic action affords a sensitive criterion of change taking place in biological material, tests emphasising enzymic differences appear to afford means for differentiating sewage liquors at different stages of treatment.

A survey of the literature on enzymes, their activation and inhibition, nitrification and B. coli organisms showed that by maintaining aerobic conditions at 37° and a pH range of 6-8 it was likely that by the use of inhibitors such as nitrites tests could be devised to differentiate between sewage and nitrified effluent.

EXPERIMENTAL

The simplest type of Barcroft respirometer with paraffin, coloured with Sudan III dye in the manometer, was used. A roll of filter paper moistened with 20 *per cent.* potassium hydroxide (0·4 ml.), was placed in the small absorption tube in each cup. Saturated mercuric chloride (6·5 ml.) was placed in one cup and the liquor (5 ml.) under test in the other. The procedure of Dixon[3] was followed, the thermostat being maintained at 37°.

The respirometer was shaken for 30 minutes with the cocks open, the motor stopped, the cocks closed and the "zero" readings taken on the manometer. The motor was restarted and manometer readings taken at definite time intervals. Moisture in the capillaries was obviated by Murray's procedure[4].

Experiments were carried out with samples of sewage, tank effluent and nitrified effluent mainly from the Cydna Sewage Works of the Johannesburg City Council. Further samples from Johannesburg, Germiston, Potchefstroom and Springs were also investigated. With satisfactory effluents manometer differences (relative oxygen uptake), plotted against time, gave straight lines with small slopes even after a few days (Fig. 1). This result seems to constitute one of the criteria of a satisfactory effluent.

With samples of sewage or tank effluent, however, irregular graphs with considerably increased slopes were obtained (Fig. 2). This was also found for mixtures of sewage and effluent. Experiments with samples of tank effluent diluted with tap water indicated that with short periods of shaking (up to six hours) it was not possible to distinguish between diluted tank effluent and nitrified effluent; in both cases straight line graphs with small slopes were obtained. When experiments were carried out over longer periods, however, ogee (S-type) curves were obtained (Fig. 3). The sudden increase in slope of the curve after a certain lag period was well emphasised up to a dilution of 1 in 5 but to a lesser extent from 1 in 5 to 1 in 10.

This indicated that after a time which varied with each sample, enzymes were produced in sufficient concentration to initiate and catalyse the reaction indicated by the steep portion of the curve. After a further period the slope of the curve decreased, indicating a further change in the general nature of the reactions.

Some of Adeney's results[5] for the rates of consumption of dissolved oxygen by the fermentable impurities in a sample of sewage liquor and in 20 *per cent.* mixtures of the same sewage with fresh water and sea water, were plotted. According to Adeney the initial portion of each curve represented the biochemical action set up by saprophytic bacteria and the final portion the nitification stage. The ogee curves (Fig. 3) obtained may therefore be interpreted as the stage of initiation during which enzymes were produced to catalyse subsequent saprophytic action (represented by a straight line with a small slope), a second stage of rapid biochemical action induced by

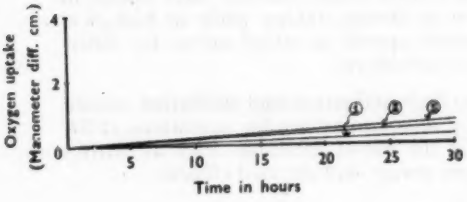

Fig. 1

Primary and secondary effluents

(1) Cydna sand filtered effluent.
(2) Rondebult final effluent (Germiston).
(3) Cydna primary effluent.

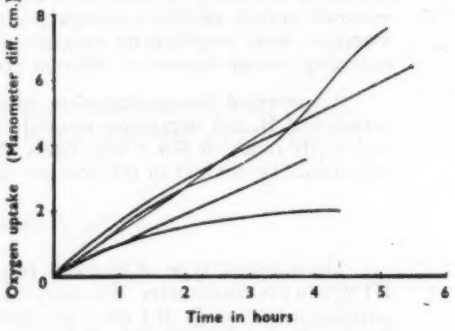

Fig. 2

Cydna Tank effluents

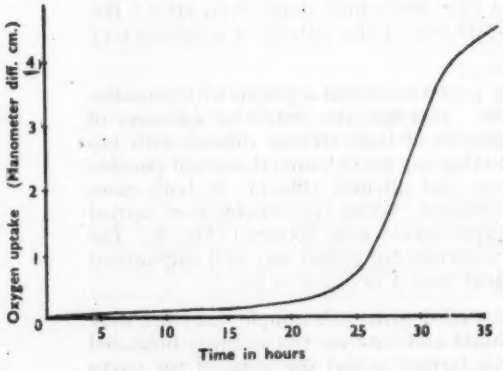

Fig. 3

Cydna tank effluent: tap water (1 : 2)

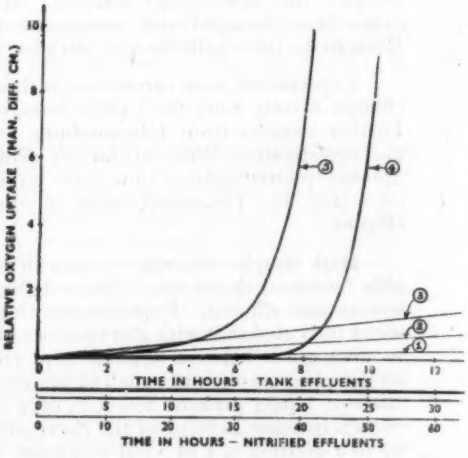

Fig. 4

Nitrified effluents: upper scale—1, 2
lower scale—3

(1) Cydna P.E., sand filtered, 5 ml. + 1 ml. McC. broth (single strength) + 1 ml. 5 per cent. sodium nitrate solution.
(2) Cydna P.E., 5 ml. + 1 ml. McC. broth (single strength) + 1 ml. 5 per cent. sodium nitrate solution.
(3) Cydna P.E. sand filtered, 5 ml. + 1 ml. McC. broth (single strength) + 1 ml. 5 per cent. sodium nitrate solution.
(4) Snap sample Cydna T.E.
T.E.: tap water 1 in 10, 5 ml. + 1 ml. McC. broth (single strength) + 1 ml. 5 per cent. sodium nitrate solution.
(5) As for 4 but average 24 hourly sample of Cydna T.E. used.

saprophytic organzims catalysed by enzymes (represented by a straight line having a considerable slope) and a third stage of nitrification represented by a straight line of lesser slope.

A well nitrified effluent itself or diluted should therefore give a straight line graph with a small slope. Thus by emphasising the second stage it was possible to distinguish dilutions of tank effluent greater than 1 in 5.

The addition of McConkey Broth[6] (double strength, 0·5 ml.) to samples of diluted tank effluent greatly reduced the induction period and saprophytic activity increased even in dilutions as great as 1 in 30. With nitrified effluents somewhat similar curves were obtained. Addition of 5 *per cent.* sodium nitrite (1 ml.), however, inhibited this reaction with nitrified effluents and the usual straight line graphs with small slopes were obtained for dilutions up to 1 in 30. Fig. 4 illustrates the distinction between nitrified effluents and diluted sewages and also the mechanism of the test.

Beyond a dilution of 1 in 30, disodium hydrogen phosphate being added, results were not reliable. Ammonium chloride, ammonia and potassium chlorate were tried without success as inhibitors. The most consistent results were obtained with McConkey Lactose Broth[6] (with sodium tauroglycocholate replacing sodium taurocholate) and sodium nitrite.

Improved test. The sensitivity of the test was subsequently improved by the use of 0·5 *per cent.* sterile sodium tauroglycocholate (1 ml.) in place of McConkey Broth. In this way it was possible to distinguish between a tank effluent diluted to 1 in 80 with tap water and a secondary effluent which had been filtered through 24 inches of sand (particle diameters from 0·2 to 2·0 mm.). The test was normally completed in less than 24 hours although shaking should be continued for 24-36 hours.

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THE NATURE OF MOLYBDENUM BLUE SOLUTIONS

by

R. ARNOLD and SHEILA M. WALKER

OPSOMMING

Molybdeenblou word berei deur elektrolitiese reduksie van molybdeensuuroplossings gevolg deur ekstraksie met butanol. In waterige oplossings van die produk word 'n waarde van 2·0 vir die verhouding van ses-valente tot vyf-valente molybdeen gevind. Verandering van die tyd van elektrolise lewer geen teken van enige reduksie trap tussen Mo^{VI} en Mo^{V} nie; dieselfde reduksie-trap word in oplossings van molybdeenblousfosfaat gevind. Konduktometriese en potensio-metriese titrasies toon dat die blou verbinding in waterige oplossing 'n matige swak suur is, waarin die aantal molybdeenatome per suurgroep met verdunning verander, vermoedelik as gevolg van kondensasie.

SUMMARY

Molybdenum blue is prepared by electrolytic reduction of molybdic acid solutions followed by extraction with butanol. The ratio of hexavalent to pentavalent molybdenum in aqueous solutions of the product is found to be 2·0. Variation in the time of electrolysis gives no indication of any other stage intermediate between Mo^{VI} and Mo^{V}; the same stage of reduction is found in solutions of molybdenum blue phosphate. Conductometric and potentiometric experiments indicate that the blue compound exists in aqueous solution as a moderately weak acid in which the number of molybdenum atoms per acid group varies with dilution, presumably due to condensation.

Reduction of slightly acid solutions containing hexavalent molybdenum yields as a first product a blue substance known as molybdenum blue. This substance appears to have been prepared in 1805 [1] and was analysed by Berzelius [2] in 1826. Since then many workers have investigated the compound, and its derivatives have found analytical applications. Nevertheless neither the composition nor the nature of molybdenum blue has been established with any certainty and a wide variety of empirical formulae have been reported [3]. As a result of more recent work [4] the term molybdenum blue must now be taken to refer to a number of closely related compounds rather than to a single substance, but it is not yet clear how many different substances of this type can exist. The possibility even arises of a continuous series of compounds intermediate between hexavalent and pentavalent molybdenum.

Since molybdenum blue is intermediate between Mo^{VI} and Mo^{V}, empirical formulae have usually been reported in the form $Mo_nO_{3n-1}\ xH_2O$, where n may have any value greater than 2. In earlier investigations the blue substance was isolated in as pure a form as possible and the molybdenum content of the product determined. That the results obtained have been discordant is not surprising, since the blue is colloidal in nature and consequently difficult to purify; furthermore, the percentage of molybdenum is not very sensitive to variations in the formula (e.g. 68·4 and 68·1% Mo for Mo_5O_{14} and Mo_6O_{17} respectively). A more satisfactory method of establishing a formula for molybdenum blue was employed by Lautié [5]. In this method a sample of the solution of the blue is titrated with potassium permanganate solution, a second sample then reduced to the trivalent stage in a Jones reductor

and again titrated with permanganate. If the two titration figures, for the same volume of solution, are T_1 and T_2 respectively, then:

$$T_2/T_1 = 3x \text{ (total molybdenum content)}/(Mo^V \text{ content}) \quad (1)$$

$$\text{or } T_2/T_1 = 3/2\ n \quad (2)$$

where n is the index in the formula Mo_nO_{3n-1}, the empirical formula of the parent oxide from which the blue is derived.

Lautié[5] prepared molybdenum blue by the action of sulphur dioxide on solutions of molybdic acid; for the titration ratio he obtained the value 7·5, corresponding to Mo_5O_{14}. Subsequently Treadwell and Schaeppi[6] applied the same analysis to samples prepared by electrolytic reduction and obtained a titration ratio of 9·0, corresponding to Mo_6O_{17}. In the present investigation essentially Treadwell and Schaeppi's method of preparation has been combined with Lautié's method of purification, and the analysis repeated. Conductometric and potentiometric titrations of the product with alkali have also been carried out.

EXPERIMENTAL

Preparation of molybdenum blue. Solutions containing about 8·5 g. per litre of molybdic acid ($MoO_3.H_2O$) and 0·05 M with respect to sulphuric acid, were electrolysed at 0·5 amp. using a mercury cathode and a platinum anode. With 600 ml. samples, optimal yields were obtained after about 4 hours. From the resulting deep blue solutions the product was extracted with butanol in an atmosphere of nitrogen after the sulphuric acid concentration had been increased to 0·2 M. The extract was washed with small quantities of air-free water until the washings were sulphate-free, some of the blue being lost in this process. Finally the butanol was evaporated under nitrogen at low pressure.

Molybdic acid was soluble to some extent in butanol, the partition coefficient between butanol and dilute sulphuric acid being about 0·2. Only traces of pentavalent molybdenum were, however, extracted by butanol.

Preparation of molybdenum blue phosphate. Samples of this derivative were prepared by the method of Treadwell and Schaeppi[6] in which molybdenum blue sulphate is first produced, by reducing molybdic acid in the presence of a relatively high concentration of sulphuric acid. The sulphate differs from the ordinary blue in that dilution with water destroys the blue colour, a red-brown appearing instead. The blue colour of the diluted solution may be restored by addition of phosphate ions.

A portion of a 0·2 M solution of molybdic acid in 4·5 M sulphuric acid was reduced in a Jones reductor and added to the remainder of the unreduced solution. The resulting blue solution was diluted 4-5 times with water, sodium phosphate (0·4 g.) added and the solution heated for 24 hours on a water bath. The blue compound was extracted with ether and the latter distilled off.

Analysis. Immediately after preparation the sample of blue was dissolved in boiling water and the solution cooled under nitrogen. An aliquot was added to ferric alum solution, heated, cooled and titrated potentiometrically with permanganate. In order to determine the total molybdenum content a second (smaller) aliquot was reduced with zinc in a Jones reductor, the reduced solution being titrated in the same way after treatment with excess of ferric alum.

Conductivity measurements were carried out with a simple Wheatstone bridge circuit using an electrically operated 1,000 cycle tuning fork as source of alternating current and a telephone receiver as detector. Conductometric and potentiometric

titrations were done with carbonate-free sodium hydroxide solution under a nitrogen atmosphere.

The results of a series of experiments in which the time of electrolysis was varied are shown in Fig. 1. The original concentration of the solutions used varied from 4·6 to 6·2 g. Mo per litre. The actual times of electrolysis have therefore been adjusted to allow for this variation, the abscissa representing 5 times actual time of electrolysis/molybdenum concentration, the factor 5 being chosen because in most cases the concentration employed was close to 5·0 g. molybdenum per litre.

For times of electrolysis of 2-5 hours the titration ratios were close to 9·0, corresponding to the parent oxide Mo_8O_{17} (eqn. (2)). The higher ratios obtained for shorter times of electrolysis may have been due to the presence of unreduced molybdic acid which was partially extracted by butanol. The ratio fell steeply after about 6 hours electrolysis when the solutions become brown, indicating complete reduction to the pentavalent stage. These results gave no positive indication of any blue compound other than that corresponding to the hypothetical oxide Mo_8O_{17}, associated with variable amounts (*ca.* 20 *per cent.*) of water.

A similar analysis was carried out on the phosphate complex of molybdenum blue. Two samples were prepared using different ratios of Mo^{III} and Mo^{VI} solutions and the resulting products analysed, giving the following results:

	Sample 1	*Sample* 2
Ratio of total Mo to Mo^{III} used in preparation	7·5	9·0
Ratio found by analysis of product	9·01	8·83

Thus molybdenum appeared to be present in the same reduction stage here as in the simple product. Treadwell and Schaeppi[6] reached the same conclusion on the basis of colourimetric measurements.

The results of conductometric titrations with alkali of aqueous solutions of molybdenum blue are shown in Fig. 2. The curved nature of the graphs, characteristic of moderately weak acids, made accurate estimation of the end points difficult, except perhaps at the lowest concentration. As the concentration was changed from 12·3 to 1·24 g. blue per litre, the equivalent amount of alkali required for neutralization increased, the range being from about 0·17 to 0·21 equivalents per g. atom of molybdenum. Thus at these concentrations there are about five molybdenum atoms per acid group, the number tending to decrease with dilution. Somewhat in keeping with this, Treadwell and Schaeppi[6] found 3 molybdenum atoms per acid group at the still lower concentration of 0·50 g. molybdenum blue per litre.

Potentiometric titration (glass electrode) gave a similar result, about 0·2 equivalents of alkali being required per g. atom of molybdenum for a solution containing 3·6 g. blue per litre. Beyond this first end point, further addition of alkali gave pH values which fell slowly to about 6 after each addition of alkali. Slow titration finally gave a sharp rise in pH at about 2 equivalents of alkali per g. atom of molybdenum. This corresponded to the final breakdown of the molybdenum blue, a brown compound, probably $MoO(OH)_3$, being precipitated. It is probable that molybdate ions were also formed in the resulting solution.

DISCUSSION

The fact that a number of different molybdenum blues exist has now been established, particularly by Glemser and Lutz[4]. These authors showed that, apart from blue anhydrous oxides, the hydrated blue oxides fall into two classes, crystalline

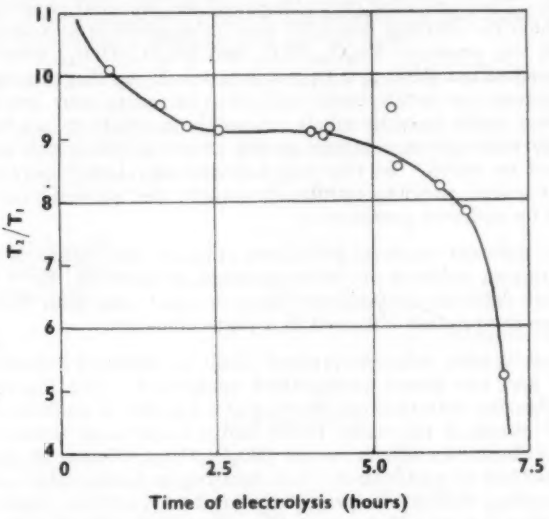


Fig. 1. Effect of time of electrolysis on the oxidation state of molybdenum blue.

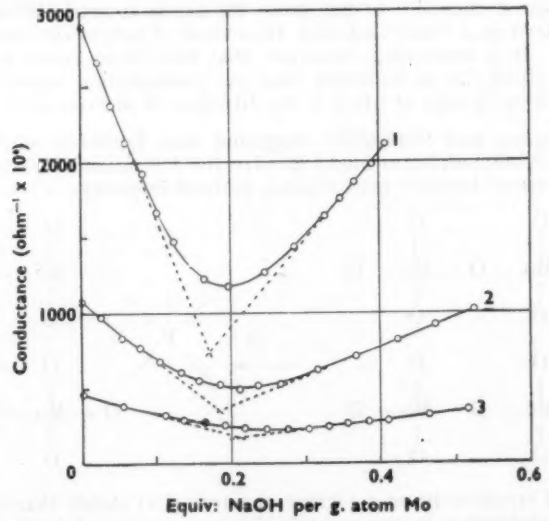


Fig. 2. Conductometric titrations of molybdenum blue solutions with alkali.

1. 12·48 g. molybdenum blue per litre, titrated with 0·810 N NaOH.
2. 4·98 g. molybdenum blue per litre, titrated with 0·405 N NaOH.
3. 1·24 g. molybdenum blue per litre, titrated with 0·123 N NaOH.

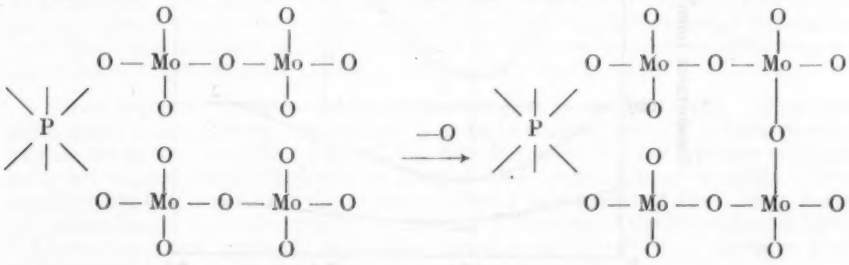
and amorphous. They prepared crystalline products by solid phase reactions or by reactions in which the starting materials were in suspension in aqueous media. By X-ray methods the products $Mo_4O_{10}(OH)_2$ and $Mo_8O_{15}(OH)_{16}$ were characterised; a product of composition $MoO_{2\cdot60}$ x H_2O with a new X-ray diagram was also isolated but its constitution not determined. All these products were insoluble in water and the first two stable towards alkali. Amorphous products, on the other hand, are obtained by reactions in solution, giving products which are soluble in water and decomposed by alkali. In this case Glemser and Lutz[4] merely repeated the preparations of several previous groups of workers and showed that the analytical results differed for different products.

Since these different products have been obtained the questions arise: whether there is a continuous series of products intermediate between Mo^{VI} and Mo^{V}, and if not, how many different molybdenum blues do exist, and what the conditions are which determine the product obtained in a particular case.

In this investigation only one product could be obtained intermediate between molybdic acid and the brown pentavalent compound. The possibility that our method of purification extracted one fraction of a mixture is unlikely since Treadwell and Schaeppi[6] obtained the same result using a different separation procedure whereas Lautié[5] obtained a different result (Mo_4O_{11}) by a different method of reduction but the same method of purification. The deciding factor therefore seems to be the conditions prevailing during the preparation rather than the proportion in which the reducing agent is used. Unfortunately no systematic investigation of the effect of such factors as pH and concentration of reagents has as yet been carried out.

Molybdenum blue solutions are colloidal and may be purified by dialysis. It was not unexpected therefore to find from the conductometric titrations that the substance behaved as a condensed acid, the extent of polymerization varying with concentration. It is interesting, however, that this result could be obtained by titration with alkali, for it indicated that the condensation equilibrium was not rapidly changed by change of pH (c.f. the titration of dichromates).

Hein, Burawoy and Schwedler[7] suggested that formation of blue reduction products of molybdiphosphates might involve the formation of oxygen bridges by loss of oxygen atoms between neighbouring molybdenum-oxygen tetrahedra:



Although based erroneously on a picture of tetrahedral rather than octahedral co-ordination of molybdenum by oxygen, this idea may prove useful. Most preparations of molybdenum blue involve reduction of molybdic acid. This substance itself is polymerised in solution, the degree of polymerization depending on the pH[8]. Elimination of water between pairs of —OH groups no doubt occurs, and the resulting —Mo — O — Mo — links would presumably not be susceptible to reduction in the

suggested manner. Since much is still to be learned about the nature of solutions of molybdic acid, a further study of this well-known substance must be added to the many lines of investigation still required in this field.

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— NOTES —

ULTRACENTRIFUGE STUDIES ON CASTOR BEAN PROTEINS

by

F. J. JOUBERT

Castor bean contains a toxic protein, ricin, which has been studied in detail[1, 2]. Little, however, has been reported on the other proteins although Bailey[3] has obtained large crystals of castor bean globulin. Recently, viscosity measurements of acetic acid and sulphur dioxide precipitated castor bean protein were reported by Joshi and Varma[4]. This communication is concerned with fractional precipitation of the proteins of the castor bean. The major sedimenting component of the protein has been isolated in a pure form and its molecular weight determined.

RESULTS AND DISCUSSION

Castor beans (*Ricinus communis*) were shelled, minced and the oil removed with acetone and hexane. The proteins of the oil-free meal were completely extracted with 10 *per cent.* sodium chloride solution and then precipitated by adding ammonium sulphate to 85 *per cent.* saturation. The precipitated proteins were only soluble in buffers of relatively high salt concentration. Measurements in a Spinco ultracentrifuge revealed (fig. 2(a)) one major component of sedimentation constant of 12·3 Svedberg units (S.U.) and minor quantities of components of 5·1 and 1·6 S.U. (respectively designated as the $S_{12\cdot3}$, $S_{5\cdot1}$ and $S_{1\cdot6}$ components) for this preparation, in a buffer of ionic strength (I) 1·0 and pH 8·8. Kabat *et al.*[2] reported a sedimentation constant of 4·8 S.U. for ricin. The $S_{5\cdot1}$ component occurring only as a minor component, therefore, represents the toxic constituent of the castor bean.

In an attempt to separate the $S_{12\cdot5}$ component from the minor components, successive amounts of saturated ammonium sulphate were added to a 10 *per cent.* sodium chloride castor bean protein extract. Fractions A, B, C, D, E, F and G were respectively obtained at 25, 30, 35, 40, 50, 65 and 85 *per cent.* saturation with ammonium sulphate and were examined ultracentrifugally in buffer I=1·0; pH=8·8. Both fraction A (45 *per cent.* of total proteins, fig. 2(b)) and fraction B (12 *per cent.* of total proteins, fig. 2(c)) contained a single $S_{12\cdot3}$ component. Fractions C, D and E (respectively 8, 6 and 3 *per cent.* of total proteins, fig. 2(d), 2(e) and 2(f)), contained in addition to the $S_{12\cdot3}$ increasing amounts of the $S_{5\cdot1}$ and $S_{1\cdot6}$ components. Fig. 2(g) shows the sedimentation diagram of fraction F (16 *per cent.*), which consisted of a major $S_{1\cdot6}$ component with small amounts of the $S_{5\cdot1}$ and $S_{12\cdot3}$ components. Fraction G (9·0 *per cent.*) (fig. 2(h)), on the other hand, contained a single $S_{1\cdot6}$ component.

When the 10 *per cent.* sodium chloride extract was diluted with five volumes of water at 4°, about 70 *per cent.* of the extracted proteins were precipitated (fraction A_1). The residual proteins were precipitated from the supernatant of fraction A_1 with ammonium sulphate (85 *per cent.* saturation) (fraction B_1). As shown in fig. 2(i), fraction A_1 consisted of a single $S_{12\cdot3}$ component. Fraction B_1 (fig. 2(j)), however, contained a major $S_{1\cdot6}$ component and small concentrations of the $S_{5\cdot1}$ and $S_{12\cdot3}$ components.

These fractionations showed that the $S_{12\cdot3}$ component was the major component of the castor bean proteins. It was also possible to isolate a large proportion of this component in a pure form by ammonium sulphate fractionation or dilution with water. Fraction A_1, in which the $S_{12\cdot3}$ component occurred, was therefore examined further.

The solubility of fraction A_1 in borate buffer I=0·3, pH=9·0 and phosphate buffer I=0·31, pH=7·0, was 0·25 and 0·15 *per cent.* respectively. No appreciable changes in the sedimentation diagrams or sedimentation constants were noticed on lowering the ionic strength from 1·0 to 0·3. Fuerst *et al.*[5] found that the crystalline globulin of squash seed dispersed in acetate buffers at various pH levels. Similarly, fraction A_1 was readily dissolved in acetate buffer I=0·1, pH=4·0. Fig. 1(a), 1(b) and 1(c) are sedimentation diagrams at different times of a 1 *per cent.* solution of fraction A_1 in this buffer. A minor concentration of a fast sedimenting component (19·8 S.U.) was also observed (fig. 1(a)). Considerable boundary spreading with time of centrifugation was noticed (fig. 1(b)) for the $S_{19\cdot8}$ component, probably due to the heterogeneous nature of this component. In fig. 1(b) and 1(c) an additional slowly sedimenting component may be seen, 56 minutes sedimentation at 250,000 g. failing to separate this component from the meniscus so that its sedimentation constant was considerably less than 1 S.U. Fraction A_1 was therefore severely changed in this buffer. The sedimentation constant of fraction A_1 increased slightly with decreasing concentration. Extrapolated to zero concentration, these results gave a value of 12·9 S.U. for the sedimentation constant.

Diffusion coefficients of fraction A_1 were determined at 20° by the free boundary method using the Tiselius U-tube for boundary formation, and the results were calculated by the maximum height and standard deviation methods. The two methods of calculation gave approximately the same diffusion coefficient for fraction A_1 (Table I), indicating a high degree of homogeneity[6] in accordance with the ultracentrifugal observations. The molecular weight and frictional ratio were calculated from the diffusion data of Table I and the value of the sedimentation constant at zero concentration.

TABLE I

Molecular weight of Fraction A_1

D°_{20} cm.²/sec. (max. height)	$3{\cdot}64 \times 10^{-7}$
D°_{20} cm.²/sec. (standard deviation) ..	$3{\cdot}62 \times 10^{-7}$
S°_{20} Svedberg Units	12·9
Partial specific volume (assumed)	0·74
Molecular weight	332,000
Frictional ratio	1·28

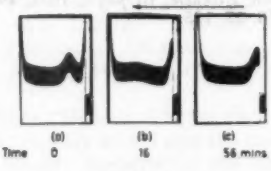

Fig. 1

Sedimentation diagrams of fraction A_1 in acetate buffer I=0·1, pH=4·0

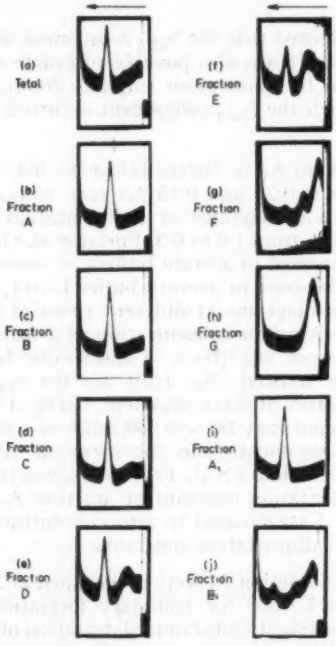

Fig. 2

Sedimentation diagrams of castor bean proteins in borate buffer I=1·0, pH=8·8

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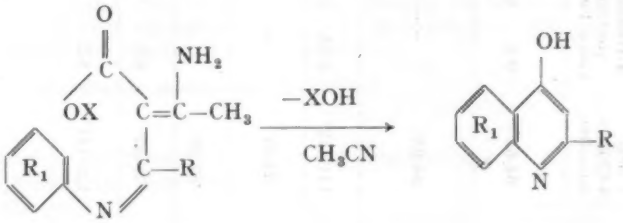
A NEW SYNTHESIS OF 2-ARYL-4-HYDROXYQUINOLINES

by

B. STASKUN

As described elsewhere[1], diaryliminochlorides condense with β-aminocrotonic esters to furnish products considered to be C-alkylated β-aminocrotonates of type (I).



(I) (II)

(X=Me or Et; R, R_1=aryl)

It is now found that the ester (I, X=Me or Et) when heated to 250-260°, undergoes cyclisation to a 2-aryl-4-hydroxyquinoline (II) in almost theoretical yield. Some evidence has been obtained that acetonitrile is produced during the reaction, but the mechanism of the conversion remains to be clarified.

EXPERIMENTAL

β-Amino-α-substituted crotonic esters. Esters of type (I) were prepared in a manner previously described[1]. In addition the following three new esters were obtained by condensing equimolecular amounts of methyl β-aminocrotonate and the appropriate iminochloride in chloroform solution at 0-4° during 3-5 days; the products were recrystallised from dilute ethanol:

Methyl β-amino-α- (N-phenyl-*o*-toluimino)-1-crotonate, m.p. 126-127°. (Found: N, 9·0 . $C_{19}H_{20}N_2O_2$ requires N, 9·1%). Methyl β-amino-α- (N-phenyl-*p*-toluimino)-1-crotonate, m.p. 182-183°. (Found: N, 9·2 · $C_{19}H_{20}N_2O_2$ requires N, 9·1%). Methyl β-amino-α- (N-phenyl-α-naphthimino)-1-crotonate, m.p. 147-148°. (Found: N, 8·2 · $C_{22}H_{22}N_2O_2$ requires N, 8·1%).

2-Aryl-4-hydroxyquinolines. Medicinal liquid paraffin (30 ml.) was heated to 250-260°, the dry crotonate (2 g.) rapidly added and the mixture kept at this temperature for 15 minutes. The ester readily dissolved in the hot paraffin on shaking and the clear yellow solution soon began to give off vapours of alcohol and acetonitrile (which were condensed and examined in a separate experiment). As the cyclisation proceeded, the sparingly soluble 4-hydroxyquinoline began to separate from solution.

TABLE I

Base $CH_3{\cdot}C(NH_2) : C{\cdot}CO_2X$ / $CR:NR_1$	4-Hydroxy-quinoline derivative	% Yield (crude) of 4-Hydroxy-quinoline	m.p. 4-Hydroxy-quinoline	m.p. 4-Chloro-quinoline	% Nitrogen				% Chlorine	
					4-Hydroxy-quinoline		4-Chloro-quinoline		4-Chloro-quinoline	
					Calcd	Found	Calcd	Found	Calcd	Found
$R=R_1=C_6H_5$; $X=CH_3$	2-phenyl	81	252-253°	64-65°	6·3	6·25	——		——	
$R=R_1=C_6H_5$; $X=C_2H_5$		83								
$R=C_6H_5$; $R_1=C_6H_4CH_3(o)$; $X=CH_3$	2-phenyl-8-methyl	81	226-228°	84-85°	——		——		14·0	13·7
$R=C_6H_5$; $R_1=C_6H_4CH_3(o)$; $X=C_2H_5$		84								
$R=C_6H_5$; $R_1=C_6H_4(OCH_3)(p)$; $X=CH_3$	2-phenyl-6-methoxy	84	308-310°	111-112°	5·55	5·8	——		——	
$R=C_6H_5$; $R_1=C_6H_3(CH_3)_2(2,4)$; $X=CH_3$	2-phenyl-6,8-dimethyl	80	228-230°	82-83°	——		——		——	
$R=C_6H_4CH_3(o)$; $R_1=C_6H_5$; $X=CH_3$	2-*o*-tolyl	82	214-215°	67-68°	——		5·5	5·45	14·0	14·1
$R=C_6H_4CH_3(p)$; $R_1=C_6H_5$; $X=CH_3$	2-*p*-tolyl	85	295-296°	79-80°	6·0	6·0	5·5	5·4	——	
$R=C_{10}H_7(\alpha)$; $R_1=C_6H_5$; $X=CH_3$	2-α-naphthyl	80	294-296°	120-121°	5·2	5·3	4·8	4·8	12·3	12·3

After cooling to room temperature, the viscous mass was treated with an equal volume of dry ether, the product filtered, washed with ether and dried (80-100°). The crude 4-hydroxyquinoline was purified either by recrystallisation from ethanol or by dissolving in boiling 1 *per cent.* sodium hydroxide, filtering from a small amount of impurity and acidifying the filtrate with dilute acetic acid.

The vapour evolved during cyclisation condensed to a slightly ammoniacal liquid. This was made just acid with dilute sulphuric acid and steam distilled. The neutral oily distillate when refluxed with 10 *per cent.* sodium hydroxide readily evolved ammonia, and the residue after acidification (sulphuric acid) was distilled to yield acetic acid (ferric chloride test). These results indicated that acetonitrile was present in the neutral oily distillate.

The corresponding 2-aryl-4-chloroquinolines were prepared in good yield by treating the pure or crude 4-hydroxyquinoline (0·5-1 g.) with phosphorus oxychloride (5-10 ml.) under reflux for 20 minutes, pouring the solution onto ice, making the mixture slightly alkaline when the 4-chloroquinoline was precipitated, and recrystallising the product from dilute ethanol.

The results are summarised in Table I.

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THE MONO-O-ACYLATION OF RESORCINOL

by

S. S. ISRAELSTAM and J. D. M. SIMPSON

Satisfactory methods are not available for the preparation of the mono-O-acyl derivatives of dihydroxyphenols. Direct acylation gives the diacyl phenol together with small amounts of the monoacyl derivative (Einhorn and Hollandt [1] and Kauffman and Kugel [2]). Knoll and Co. [3] state that monoacyl derivatives may be prepared by the metathesis at high temperature of the phenol and its diacyl derivative, whilst Gearheart and Pugh [4] reacted sodium methoxide with resorcinol and resorcinol dibenzoate at 110°. Benet [5] obtained good yields (60 *per cent.*) of resorcinol monobenzoate by boiling an alcoholic solution of the dibenzoate with an aqueous solution containing trisodium and disodium phosphates and reported that the addition of formalin increased the yield of monobenzoate to 90 *per cent.*

Repetition of Benet's experiment on the dibenzoate without the use of formalin confirmed his findings, but application of this method to resorcinol diacetate, resorcinol dicinnamate, hydroquinone diacetate, hydroquinone dibenzoate and catechol dibenzoate resulted in complete hydrolysis in ea h case. The addition of formalin gave resinous materials.

Since Chattaway [6] showed that a good yield of resorcinol diacetate was obtained from one mole of resorcinol, two moles of sodium hydroxide and two moles of acetic anhydride, the use of one mole each of anhydride and sodium hydroxide could lead to the monoacetate. This was not found to be the case, a 50 *per cent.* yield of the diacetate was obtained, half the resorcinol remaining unchanged. When, however, one mole of resorcinol and *two* moles of sodium hydroxide were treated with one mole of acetic anhydride in the cold, only resorcinol monoacetate was obtained in 70 *per cent.* yield. This may be accounted for as follows:

(i) $$C_6H_4(OH)_2+2NaOH\longrightarrow C_6H_4(ONa)_2+2H_2O$$

(ii) $$C_6H_4(ONa)_2+(CH_3CO)_2O\longrightarrow C_6H_4(ONa)(OCOCH_3)+CH_3COONa$$

Since in some experiments the diacylresorcinol was isolated it is possible that the following reactions occurred:

$$\tfrac{1}{2}C_6H_4(ONa)_2+(CH_3CO)_2O\longrightarrow \tfrac{1}{2}C_6H_4(O\cdot COCH_3)_2+\tfrac{1}{2}CH_3COONa$$

$$\tfrac{1}{2}C_6H_4(ONa)_2+\tfrac{1}{2}C_6H_4(O\cdot COCH_3)_2\longrightarrow C_6H_4(ONa)(O\cdot COCH_3)$$

That the latter reaction does occur was shown by the production of resorcinol monoacetate in 83 *per cent.* yield when resorcinol diacetate was shaken with a solution containing an equimolecular quantity of disodium resorcinate.

This modification of the Chattaway method [6] was used to prepare resorcinol monobutyrate and resorcinol monopropionate, neither of which had been prepared

before. In each case a small quantity of the diacyl resorcinol was isolated. This method was not successful for the preparation of the monoacetates of catechol, hydroquinone and orcinol, nor for the preparation of the monobenzoate and monocinnamate of resorcinol, the diesters only being formed in each case.

EXPERIMENTAL

Mono-O-acylation. Acetic anhydride (1 mole) was added to resorcinol (1 mole) in 2N sodium hydroxide (2 moles) containing crushed ice. Where the diester separated it was extracted with ether and the residual alkaline solution then acidified to yield the monoester.

Anhydride	Ester	% Yield	B.P.	Found %C	Found %H	Calcd. %C	Calcd. %H	Literature B.P.
Acetic	monoacetate	70	158-160°/ 9 mm.	63·3	5·3	63·2	5·3	283°/760 mm.[7]
propionic	monopropionate	76	168-170°/ 12 mm. 280°/626 mm.	64·9	6·1	65·0	6·1	not reported
	dipropionate	3·6	293°/626 mm.					297-8°/760 mm.[8]
butyric	monobutyrate	74	159-161°/ 9 mm.	66·5	6·6	66·65	6·7	not reported
	dibutyrate	10						195-200°/ 18 mm.[9]

Reaction between resorcinol diacetate and disodium resorcinate. On shaking resorcinol diacetate (10 g.) with a solution of disodium resorcinate (from 5·7 g. resorcinol and 80 ml. 2N sodium hydroxide) and ice (50 g.) a clear solution resulted. Acidification yielded resorcinol monoacetate (13·2 g., 83 *per cent.*).

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